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Simple and non toxic enzyme immobilization onto platinum electrodes for detection of metabolic molecules in the rat brain using silicon micro-needles

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Abstract

We report the successful fabrication of microelectrode biosensors, onto Pt wires or silicon micro-needles, based on oxidase immobilization by poly(ethylene glycol) diglycidyl ether (PEGDE). We found that biosensors made with PEGDE exhibited high sensitivity and a response time on the order of seconds. Glucose biosensors implanted in the central nervous system of anesthetized rats reliably monitored changes in brain glucose levels induced by sequential administration of insulin and glucose. PEGDE provides a simple, low cost, non-toxic alternative for the preparation of *in vivo* microelectrode biosensors. This innovative method was applied to functionalize Pt microelectrodes deposited onto silicon microprobes. Their operating parameters were characterized and validated *in vivo*.

Keywords: biosensor; microelectrode; silicon needle; PEGDE; glucose; *in vivo*.

1. Introduction

Implantable enzymatic biosensors are widely used for in vivo monitoring applications. They are used in neuroscience research to detect neurotransmitters and metabolites with outstanding temporal resolution [1,2]. These biosensors are usually glass capillary-based platinum microelectrodes constructed according to a common basic design [3]. However, recent studies are oriented on the development of multisensing

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probes for simultaneous monitoring of neurotransmitters or metabolites during physiological or pathological functioning of the central nervous system (CNS) [4]. Additionally, special attention must be paid to the protein immobilization procedure, because it impacts the overall biosensor performance. A wide variety of enzyme immobilization techniques are now available for biosensor construction. However, most methods involve toxic components that make potential clinical applications difficult [4,5,6]. Moreover, the chemicals used for biosensor preparation are often expensive or imply some organic synthesis [7]. Up to now, only few groups succeeded to fabricate multisite probes and applied them *in vivo* [4,5], using highly toxic glutaraldehyde for enzyme immobilization. Overall, there is a need for simple, clean and cheap methods for enzyme immobilization on biosensors that could be easily reproduced by non-expert laboratories.

In the present study, we investigated the utilization of PEGDE as a matrix for immobilizing oxidoreductases on glass capillary biosensors. PEGDE is an essential component of the redox hydrogels used for commercial glucose biosensors [8]. However, the interest of using PEGDE alone for immobilizing proteins on biosensors has never been thoroughly explored. Here, we firstly examined the possibility of several enzymes immobilization onto glass capillary microelectrodes using PEGDE alone. The biosensor principal operational parameters were explored and glucose biosensors were validated in the CNS of anesthetized rats [9]. Secondly, we produced three-electrode micro-needles by standard micromachining process, adapted PEGDE deposition procedure and locally deposited different protein layers on each electrode. Biosensors were characterized, cross-talk effects between electrodes were measured and finally, they were implanted in the brain of the anesthetized rat for validation in real biological conditions.

2. Enzyme immobilization on electrodes by PEGDE.

PEGDE reacts with the amine functional groups present in proteins to create a matrix for enzyme immobilization on the electrode surface (Fig. 1). The reaction between the diepoxide and proteins is slow at room temperature (~48 h), but can be accelerated considerably by increasing the temperature (8). We therefore set the temperature at 55 °C, which is low enough to preserve the activity of all oxidase enzymes we tested and 2h incubation of the enzyme/PEGDE mixture was sufficient to achieve immobilization in a stable membrane. We tested the PEGDE enzyme immobilization process by preparing glucose, D-aminoacid and glutamate oxidase-based biosensors. We studied the dependence of biosensor sensitivity on the amount of PEGDE added to the enzyme and showed that the sensitivity of the biosensors followed a bell-shaped curve. The optimal PEGDE concentrations for glucose oxidase, DAAO and glutamate oxidase were 20 mg/mL, 5.9 mg/mL and 78 mg/mL, respectively in enzyme solutions containing 60 mg/mL protein of interest, 30 mg/mL of BSA and glycerol at 1% in 0.01 M PBS. The enzyme membrane appeared as a transparent film insoluble in water.

The characteristics of our biosensors were determined and compared with those obtained after fixation with glutaraldehyde, one of the most widely used fixative reagents for enzymatic biosensor fabrication. The response times of the PEGDE biosensors were similar to those obtained using glutaraldehyde. The Km values were generally similar between the two methods. The PEGDE biosensor sensitivities were generally higher than those observed with glutaraldehyde fixation [9].

3. *In vivo* validation

We tested the utility of the PEGDE biosensors in applications requiring *in vivo* brain implantation. We implanted glucose biosensors in the frontal cortex of anesthetized rats. Basal brain glucose concentrations were 0.68 ± 0.15 mM (n = 8). Injection of insulin induced a steady decrease in oxidation current from the

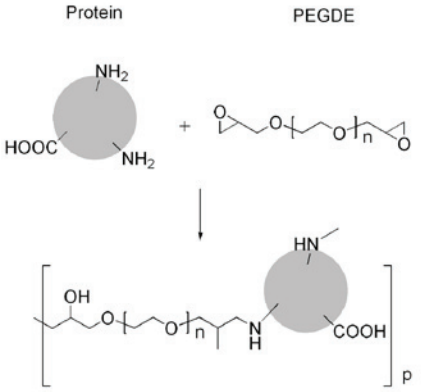

Fig. 1. General reaction scheme of protein with poly(ethylene glycol) diglycidyl ether. All protein molecules (enzyme, bovine serum albumin, etc.) contain amino- and carboxyl groups that may react with the 2 epoxides of PEGDE, resulting in immobilization.

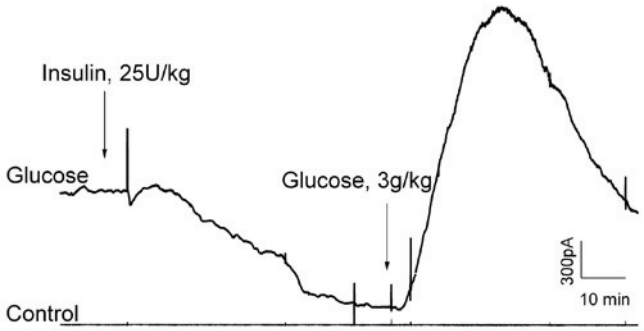

Fig. 2. Biosensor recordings in vivo. Glucose monitoring using a PEGDE/glucose oxidase biosensor implanted in the frontal cortex of an anesthetized rat. Signal detected by a glucose microbiosensor (bold line) was compared to that recorded by a control (BSA) biosensor implanted on the contra-lateral side of the brain (thin line).

glucose microbiosensor, but not from the control electrode. Injection of glucose 1 h after insulin administration quickly raised the brain glucose signal above its original value (Fig. 2). The extracellular brain glucose concentration 1 h after insulin injection was 0.19 ± 0.05 mM, and the concentration rose to 1.1 ± 0.2 mM after glucose injection [9]. Calibration experiments performed after electrode removal from the brain revealed that our biosensors were still functional after *in vivo* implantation, with some even displaying a slight increase in sensitivity. The fact that we could detect glucose changes with our glucose oxidase/PEGDE biosensors therefore indicates that our method is well suited for *in vivo* brain glucose monitoring.

4. Biosensor on silicon microneedle.

The microprobes were fabricated starting from 250 µm (100) silicon wafer. The first mask was transferred to a previously grown thermal oxide (2 µm) to form the needle contour in the front side and the second mask was transferred to oxide in the back side to mark further membrane position versus needle. The subsequent HF etch of double side oxide was performed. The metallization (20nm Ti and 150nm Pt) was patterned by a lift-off process using AZ5214 photoresist to design the three-electrode probe. Negative SU8- 2000.5 photoresist was used as top passivation. It was opened at the recording sites and bonding pads by photoresist development. To determine the needle shape, the first DRIE etch was performed on the front side, followed by second one on the back side to define the needle thickness (50µm) (Fig. 3). During etching the Pt and SU8 surfaces were protected by photoresist. Finally, the probes were cut down by diamond saw and packed with custom-made printed circuit boards.

Prior to enzyme immobilization, electrodes were cleaned by cyclic voltammetry treatment in 1M H_2SO_4 solution. Glucose oxidase local deposition onto Pt electrode was achieved with chitosan assistance. Chitosan is natural sugar-based polymer, soluble at acid pH and insoluble in neutral solution. Application of a negative potential (-1.3 V) to the working electrode provokes chitosan precipitation onto electrode, accompanied with enzyme and PEGDE entrapment (Fig. 3). Chitosan contains functional amine groups in its structure, and PEGDE performs reliable attachment of proteins to the chitosan layer while preventing enzyme leaching from the deposited membrane. We showed that enzyme

immobilization involving chitosan decreases the sensitivity of biosensor toward its substrate, compared to immobilization by PEGDE alone. Finally, we carried out the preliminary tests for monitoring glucose concentrations *in vivo* with silicon micro-needles. The first Pt electrode was modified by Glucose oxidase containing layer while two others were covered only with chitosan/PEGDE layer. We obtained monitoring profile similar to that recorded by glass capillary-based microbiosensors (Fig. 2). Basal brain glucose concentrations, glucose concentrations 1 h after insulin injection and after glucose injection were estimated at 0.52mM, 0.18mM and 0.88mM respectively. Obtained glucose concentration values are close to those estimated by glass capillary-based biosensors. These results show that amperometric biosensors prepared with oxidases immobilized onto silicon micro-needles using PEGDE can reliably monitor the extracellular glucose concentration in the CNS of laboratory animals.

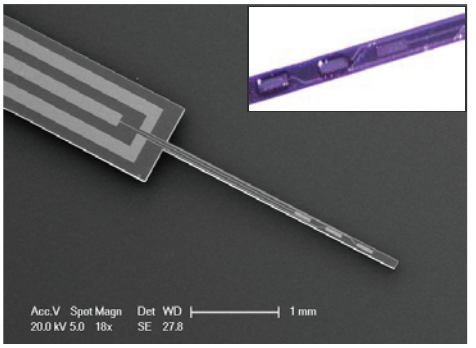

Fig. 3. SEM image of a micromachined silicon microneedle with 3 Pt electrodes. Insert shows enzyme immobilization on the Pt electrodes by PEGDE with chitosan-assisted local deposition.

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